Ubiquitylation of a Melanosomal Protein by HECT-E3 Ligases Serves as Sorting Signal for Lysosomal Degradation[□]

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The production of pigment by melanocytic cells of the skin involves a series of enzymatic reactions that take place in specialized organelles called melanosomes. Melan-A/MART-1 is a melanocytic transmembrane protein with no enzymatic activity that accumulates in vesicles at the trans side of the Golgi and in melanosomes. We show here that, in melanoma cells, Melan-A associates with two homologous to E6-AP C-terminus (HECT)-E3 ubiquitin ligases, NEDD4 and Itch, and is ubiquitylated. Both NEDD4 and Itch participate in the degradation of Melan-A. A mutant Melan-A lacking ubiquitin-acceptor residues displays increased half-life and, in pigmented cells, accumulates in melanosomes. These results suggest that ubiquitylation regulates the lysosomal sorting and degradation of Melan-A/MART-1 from melanosomes in melanocytic cells.

INTRODUCTION

Ubiquitylation, the process by which ubiquitin (Ub) is conjugated to Lys side chains of proteins, has emerged as the mechanism by which most intracellular proteins are targeted for lysosomal and proteasomal degradation, the two major proteolytic systems of the cell. Whereas most cytoplasmic proteins are degraded by the proteasome, a multicatalytic protease complex, cell surface proteins are generally targeted to the lysosomes. The type of ubiquitylation required for the targeting of substrates to each of these proteolytic systems differs (Hicke, 2001; Weissman, 2001). Proteins targeted to the proteasome generally carry multi-Ub chains, whereas those targeted for lysosomal degradation are modified by one to four Ub moieties. In both cases, one or several Lys side chains can serve simultaneously as Ub acceptors.

Substrate ubiquitylation is mediated by Ub ligases, also called E3 Ub ligases (Weissman, 2001). Among the many E3 Ub ligases, the family of homologous to E6-AP C-terminus (HECT)-E3 Ub ligases regrouping the yeast Rsp5p and the mammalian homologues NEDD4, AIP4/Itch (hereafter called Itch), and Smurf, has been shown to ubiquitylate membrane proteins, and, in some instances, to induce their degradation (Ingham *et al.*, 2004). HECT-E3 Ub ligases are

characterized by the presence of a C-terminal HECT domain that contains the active site for Ub transfer onto substrates. In addition, they contain an N-terminal lipid-interacting C2 domain and protein-protein-interacting WW domains.

Melanocytes are specialized cells endowed with the capacity of producing the pigment melanin. The biosynthesis of melanin from the amino acid tyrosine produces reactive O₂ species and other cytotoxic by-products and is thus confined to membrane-bound specialized lysosome-related organelles called melanosomes (Raposo and Marks, 2002). Melanosomes are morphologically classified into four maturation stages, ranging from early, nonpigmented stage I/premelanosomes to mature, heavily pigmented stage IV melanosomes that can be transferred to neighboring keratinocytes. The biogenesis of melanosomes entails a complex maturation process, which involves the formation of a luminal matrix, onto which melanin is later deposited, and the sorting of various melanogenic enzymes.

Melan-A/MART-1 (abbreviated hereafter as Melan-A) is a protein specifically expressed by melanocytic cells (Coulie *et al.*, 1994; Kawakami *et al.*, 1994; Romero *et al.*, 2002). Although it has been widely studied as target in the development of antimelanoma vaccines, its cellular function has remained elusive. Melan-A is a palmitoylated integral membrane protein of 118 amino acids with a short amino-terminal luminal domain and a longer carboxy-terminal cytoplasmic domain (Rimoldi *et al.*, 2001; De Maziere *et al.*, 2002). The protein does not possess any detectable enzymatic activity and has not been linked to any of the numerous genetic defects that affect skin pigmentation (Kawakami *et al.*, 1994). Using immunoelectron microscopy, we have previously shown that Melan-A predominantly accumulates in small vesicles at the *trans*-Golgi network (TGN) and in early stage melanosomes (stage I and II), although it also was present in mature melanosomes as well as late endosomes and lyso-

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Abbreviations used: HECT, homologous to E6-AP C-terminus; MVB, multivesicular body; Ub, ubiquitin.

somes (De Maziere *et al.*, 2002). In the latter, Melan-A was associated with internal membrane profiles.

In this work, we demonstrate that Melan-A interacts with the E3 Ub ligases NEDD4 and Itch and is ubiquitylated in melanocytic cells. Furthermore, we show that ubiquitylation of Melan-A is required for degradation of the protein by lysosomes in pigmented cells. These results implicate E3 Ub ligases in the sorting of a melanosomal protein.

MATERIALS AND METHODS

Cells and Transfections

The highly pigmented MNT-1 melanoma cell line was a gift from P.-G. Natali (University La Sapienza, Rome, Italy). The amelanotic (Melan-A, tyrosinase, and gp100 negative) SK-Mel-37 and NA8-MEL melanoma cells were a gift from Y.-T. Chen (Ludwig Institute for Cancer Research, New York, NY) and F. Jotereau (Biology Institute, INSERM U463, Nantes, France), respectively. Melanoma cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). Human embryonic kidney (HEK)293T cells were maintained in DMEM/10% FCS. Transfections were performed using LipofectAMINE 2000 reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. For *in vivo* ubiquitylation assays, HEK293T cells were plated in 100-mm plates and cotransfected, as indicated, with Melan-A plus or minus hemagglutinin (HA)-tagged Ub, NEDD4-1-, NEDD4-2-, or Itch-encoding plasmids keeping a constant amount of total plasmid DNA by adding, when necessary, empty pcDNA3 vector. The Ub plasmid was used at one-fifth the amount of Melan-A plasmids. Itch- and NEDD4-overexpressing Melan-A-transduced SK-Mel-37 cells were obtained by transfection of plasmids by using FuGene (Roche Diagnostics, Mannheim, Germany). Transfected cells were subjected to Geneticin (G418) selection, and the pools of resistant cells were used for subsequent experiments (~30% of these cells overexpressed the respective ligases). A neomycin-resistant culture of cells transfected with empty pcDNA3 vector was generated in parallel as negative control.

Plasmids

The plasmid pcDNA3 served as vector for the transfection of all Melan-A constructs. Melan-A coding sequence was amplified by polymerase chain reaction (PCR) from a plasmid containing the full-length cDNA (a gift from P. Coulie, University of Louvain, Brussels, Belgium) by using the primers 5'-GCGCTAGCGGATCCATGCCAAGAGAAGATGCTCA-3' (sense) and 5'-GCGCGAATTCTAGATTAAGGTGAATAAGGTGGTG-3' (antisense) and inserted into the EcoRI/BamHI sites of pcDNA3. Plasmids encoding Melan-A with single K to R substitutions, Melan-A^{K3-6R} and Melan-A^{K1-6R} were obtained using the PCR-based QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), following the manufacturer's protocol. Carboxy-terminal HA-tagged versions of Melan-A were obtained by PCR technique by using an antisense primer incorporating an HA sequence. Plasmids used to produce recombinant lentivirus for expression of various Melan-A proteins (wild-type Melan-A and Melan-A^{K1-6R}, with and without C-terminal HA tag) were obtained by subcloning EcoRI/BamHI fragments into corresponding sites of a lentiviral vector based on pHR' (a gift from R. D. Iggo, Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland) (Naldini *et al.*, 1996). The vector contains an EF1 α promoter followed by an SV40-puromycin acetyl transferase cassette for antibiotic selection. The cytosolic domain of Melan-A (Melan-A₅₀₋₁₁₈) was PCR amplified and cloned into the BamHI-XhoI sites of pGEX4T-1, producing an in-frame fusion with glutathione S-transferase (GST). Human Itch was cloned by PCR amplification of cDNA prepared from BB64-RCC cells and inserted into the NheI-BamHI sites of the pEGFP plasmid, thereby replacing the coding sequence of enhanced green fluorescent protein (EGFP) by the one of Itch. The sequences of all constructs were confirmed by DNA sequencing. The plasmid encoding a catalytically inactive Itch, Itch^{C830A}, was a gift from A. Angers (Department of Biological Sciences, University of Montréal, QC, Canada) (Angers *et al.*, 2004). The plasmid coding for HA-tagged Ub was a kind gift of K. Burns (Biochemistry Institute, University of Lausanne, Lausanne, Switzerland). The plasmids encoding NEDD4-1 and the catalytically inactive variant NEDD4-1^{C867S} were generously provided by O. Staub (Department of Pharmacology and Toxicology, University of Lausanne) (Blot *et al.*, 2004).

Antibodies and Other Reagents

The monoclonal antibodies A103 (a gift from E. Stockert, Ludwig Institute for Cancer Research) and M2-7C10 (a gift from Y. Kawakami and S. A. Rosenberg, National Institutes of Health, Bethesda, MD) against Melan-A have been described previously (Chen *et al.*, 1996; Kawakami *et al.*, 1997; De Maziere *et al.*, 2002). A103 was used in a Sepharose-conjugated form for immunoprecipitations, whereas M2-7C10 was used for Western blot detection. The anti-FLAG antibody M2 was from Sigma-Aldrich (St. Louis, MO); unconjugated (mouse) and immobilized (rat) anti-HA monoclonal antibody were from

Covance (Berkeley, CA) and Roche Diagnostics, respectively; rabbit polyclonal anti-actin antibody was from Sigma-Aldrich; anti-Itch, anti-Cbl, and anti-Lamp-1/CD107a monoclonal antibodies were from BD Biosciences Pharmingen (San Diego, CA); mouse monoclonal antibodies TA99 (a gift from A. Houghton, Memorial Sloan-Kettering Cancer Center, New York, NY) and NK1-beteb (Vennegoor *et al.*, 1988) were used to detect TRP-1 and gp100, respectively. The anti-NEDD4 rabbit serum, raised against the WW domains of human NEDD4-1 but cross-reacting with NEDD4-2, was generously provided by O. Staub (Staub *et al.*, 1996). The anti-Ub antibody (clone FK2) was purchased from Affiniti/BIOMOL (Exeter, United Kingdom). Secondary reagents used were peroxidase-conjugated streptavidin, anti-mouse IgG, and anti-rabbit IgG (Amersham Biosciences, Piscataway, NJ); Alexa Fluor⁴⁸⁸ goat anti-mouse IgG (Molecular Probes, Eugene, OR); and Cy3-conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA). The proteasome inhibitor lactacystin was obtained from BIOMOL and *N*-benzoyloxy-carbonyl (Z)-Leu-Leu-leucinal (MG132) was from Calbiochem (San Diego, CA). Inhibitors were used at a final concentration of 50 μ M (lactacystin) and 25–50 μ M (MG132). NH₄Cl, chloroquine, bafilomycin A1, and concanamycin A (all from Sigma-Aldrich) were used at 30 mM, 50–200 μ M, 100 nM, and 20 nM, respectively. All inhibitors were dissolved in water, except MG132 (dimethyl sulfoxide).

Recombinant Lentiviruses and Infections

For lentivirus production, the lentiviral vector plasmid (20 μ g) and second generation packaging plasmids (5 μ g of pMD2-VSVG and 15 μ g of pCMV-R8.91) (Naldini *et al.*, 1996) were cotransfected into HEK293T cells as detailed (www.tronolab.unige.ch). Lentivirus-containing supernatants were collected 48 h after transfection, 0.2 μ m filtered, and snap-frozen at -70° C. For infections, SK-Mel-37 and MNT-1 cells were plated in six-well plates (10⁵ cells/well) 24 or 48 h before infection, respectively, and virus-containing supernatant was added to attain 10–100% infection. Puromycin selection (5 μ g/ml for MNT-1 and 2.5 μ g/ml for SK-Mel-37) was applied 48 h postinfection. Transduced cells were used within few weeks from infection. As negative controls, MNT-1 cells were transduced with an "empty" lentivirus (virus made with the transducing vector devoid of insert).

Silencing of Itch and NEDD4

Melan-A-transduced SK-Mel-37 cells were transfected with small interfering RNA (siRNA) duplexes (200 nM; Dharmacon, Chicago, IL) targeting Itch, NEDD4, or, as control, tripeptidyl peptidase II (TPP II), using Oligofectamine (Invitrogen) according to the manufacturer's protocol. The mRNA target sequences of the Itch, Nedd4, and TPP II siRNA duplexes were 5'-AAG-GAAAAUAAGAAGAAUUGG-3', 5'-AAUCCAAAGUGGAAUGAAGAA-3', and 5'-AACUACAGCCUUUGCCUACAG-3', respectively. Mock-transfected cells were included as additional control. Transfected cells were incubated for 48 h in Opti-MEM 10% FCS and then collected and lysed in 1% Triton X-100, 20 mM Tris-HCl, pH 7.6, and 150 mM NaCl supplemented with Complete protease inhibitor cocktail (Roche Diagnostics).

Immunoprecipitation

A preparative immunoprecipitation was performed with MNT-1 cells (10⁸) expressing HA-tagged Melan-A and nontransduced MNT-1. Cells were lysed (10 vol buffer/vol cell pellet) in 1% Triton X-100, 150 mM NaCl, and 50 mM Tris-HCl, pH 7.5, supplemented with Complete protease inhibitor cocktail (Roche Diagnostics), *N*-ethylmaleimide (NEM; 10 mM) and iodoacetamide (IAA; 1 mM) for 30 min on ice, followed by centrifugation. The supernatant was precleared with quenched Sepharose-CNBr for at least 2 h at 4°C, followed by immunoprecipitation with Sepharose-conjugated A103 mAb overnight (o.n.) at 4°C. After seven washes with lysis buffer, immunocomplexes were eluted by boiling in Laemmli buffer and resolved on a 12% acrylamide gel under reducing conditions (20 mM dithiothreitol [DTT] and 5 mM IAA). Gel was stained with Brilliant Blue G-collodial (Sigma-Aldrich) according to manufacturer's protocol. Bands of interest were cut out and processed for in-gel trypsin digestion and mass spectrometry analysis as described previously (Lévy *et al.*, 2002). For coimmunoprecipitations of NEDD4 and Itch with Melan-A in MNT-1 cells, lysates were prepared and precleared as described above. Lysates were then immunoprecipitated o.n. with Sepharose-conjugated A103 and control antibody (anti-CD8), or with anti-Itch and anti-NEDD4 antibodies together with agarose-conjugated protein G (Pierce Chemical, Rockford, IL). After washing and eluting immunocomplexes as described above, aliquots were separated on 12% (Melan-A) or 8% (NEDD4 and Itch) polyacrylamide gels under reducing conditions, and Western blotting was performed with appropriate antibodies according to standard procedures. For *in vivo* ubiquitylation experiments, HEK293T cells cotransfected with the indicated plasmids were collected 24 h later and lysed in radioimmunoprecipitation assay (RIPA) buffer (1% Triton X-100, 1% deoxycholate, 0.1% SDS, 150 NaCl, and 10 mM Tris-HCl, pH 7.5) containing IAA (1 mM), NEM (10 mM), and Complete protease inhibitor cocktail. In other transfection experiments, HEK293T cells were collected 48 h after transfection. Lysates were processed for immunoprecipitation and Western blotting as described above. When necessary, aliquots of cell lysates were processed for Western blot analysis without immunoprecipitation. Signal was detected

with an enhanced chemiluminescence detection system (Amersham Biosciences) or Super Signal West Femto (Pierce Chemical).

Metabolic Labeling

Metabolic labeling with ^{35}S -Cys and pulse-chase experiments were performed as described previously (De Maziere *et al.*, 2002), except that cells were pulsed for 30 min and lysed in RIPA buffer. For pulse-chase experiments with lysosome inhibitors, cells were chased in the presence of NH_4Cl or chloroquine. Lactacystin and MG132 were added during the labeling period and further included in the chase medium. Because the latter drugs affected incorporation of radioactivity, zero time chase points were prepared by including the appropriate drug and solvent during the labeling period.

Microscopy

Double immunofluorescence stainings were performed as described previously (De Maziere *et al.*, 2002). Confocal analyses were performed with a Zeiss Axiocvert 100 microscope coupled to a laser scanning microscope Zeiss 510 (Carl Zeiss, Jena, Germany) by using a 63 $\times$ Plan ApoChromat objective (1.4 oil). Images were acquired in the multi-track mode. Differential interference contrast images were acquired with a Leica LMIRB DC200 instrument by using plan 20 $\times$ /0.4 lenses (image size 1272 $\times$ 1017 pixels). Melan-A transduced SK-Mel-37 cells transfected with E3 ligases were photographed with an Axioplan 2 microscope (Carl Zeiss) by using Plan-Apochromat 20 $\times$ lenses, and pictures were acquired with Axiovision 4.2 software. Images were further processed with Adobe Photoshop version 6.0.

In Vitro Ubiquitylation

The GST-fused cytosolic domain of Melan-A (Melan-A_{50–118}) was expressed in *Escherichia coli* after induction with 0.8 mM isopropyl β -D-thiogalactoside. Cells from 50-ml cultures were lysed in 1.6 ml of buffer containing 800 μl of Tris-buffered saline (TBS) (20 mM Tris-HCl, pH 7.6, and 100 mM NaCl) and 800 μl of ProFound lysis buffer (Pierce Chemical). The material was purified on 250 μl of glutathione-coated beads (Pierce Chemical). The beads were washed four times in TBS and used directly in the assay, without prior elution. Proteasome-depleted cytosol from NA8-MEL cells was prepared as described previously (Lévy *et al.*, 2002). Cytosol equivalent to $\sim 3 \times 10^6$ cells was added to the beads together with 10 mM ATP, 0.2 mM DTT, 5 mM Mg-acetate, and 1 μg of biotinylated Ub (Affiniti/BIOMOL). The reaction was allowed to proceed for 3 h at 37°C and then stopped by adding 10 mM EDTA. The beads were twice washed with cold buffer (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM EDTA, and 0.1% NP-40), dried, and diluted into SDS-PAGE sample buffer containing 20 mM DTT. The samples were boiled and separated by SDS-PAGE. Where indicated, the proteasome-depleted cytosol was adsorbed on immobilized rabbit anti-NEDD4 or rabbit anti-proteasome antibodies (control depletion) for 45 min at 4°C. Adsorption was repeated twice. Supernatant was used for the ubiquitylation assay.

Pull-Down Assay

HEK293T cells (3×10^7) were lysed in 0.5% Triton X-100, 50 mM Tris-HCl pH 7.6, and 150 mM NaCl. The cell lysate was incubated with GST or GST-Melan-A (prepared as described above) immobilized on glutathione beads for 45 min at 4°C. The beads were washed three times in lysis buffer and eluted with SDS-PAGE sample buffer.

RESULTS

Melan-A Is Ubiquitylated in Melanoma Cells

In an attempt to uncover proteins interacting with Melan-A, we transduced the Melan-A-positive pigmented melanoma cells MNT-1 with a recombinant lentivirus coding for a C-terminally HA-tagged Melan-A and performed immunoprecipitation experiments with an anti-HA antibody. The use of an epitope-tagged Melan-A was motivated by the fact that the two available Melan-A-specific monoclonal antibodies are directed against neighboring epitopes within the highly conserved C-terminal region of Melan-A (De Maziere *et al.*, 2002) that is potentially involved in protein-protein interactions. The immunoprecipitated material was resolved by SDS-PAGE and stained. As shown in Figure 1A, three major bands were present in the material immunoprecipitated from transduced melanoma cells (left lane) and absent from mock-infected cells (right lane). The bands were excised, in-gel digested with trypsin, and subjected to identification by mass spectrometry. As predicted, the most prominent band, migrating at ~ 25 kDa, was identified as

Melan-A. Two additional bands of higher molecular weight (~ 30 and 36 kDa) contained peptides matching both Melan-A and Ub sequences, suggesting that Melan-A may exist in a ubiquitylated form. The presence of ubiquitylated Melan-A was confirmed in unmanipulated MNT-1 cells by the detection of Ub-containing bands in the material precipitated with anti-Melan-A but not control antibodies (Figure 1B). A similar Ub pattern was obtained when cells were lysed in SDS before immunoprecipitation, to exclude the detection of ubiquitylated proteins associated with Melan-A (Figure 1B, right). The size of ubiquitylated Melan-A forms detected in melanoma cells was consistent with the addition of 1–5 Ub moieties (the putative monoubiquitylated form was visible in MNT-1 cells at longer exposure times). Melan-A ubiquitylation also was reproduced using an in vivo ubiquitylation assay, whereby HEK293T cells (Melan-A-negative) were cotransfected with Melan-A and HA-tagged Ub (Figure 1C).

Melan-A contains six Lys residues. Four of these are present in the cytosolic domain and could thus potentially serve as Ub acceptor. Substitution of all 6 Lys or the four cytosolic Lys to Arg (Melan-A^{K1-6R} and Melan-A^{K3-6R}, respectively) abrogated Melan-A ubiquitylation in transfected HEK293T cells, as shown in Figure 1D. However, single or double Lys-to-Arg substitutions did not prevent ubiquitylation, suggesting that multiple Lys could serve as Ub acceptor (our unpublished data). Finally, ubiquitylation of Melan-A also could be reconstituted in vitro, by incubating the fusion protein GST-Melan-A_{50–118} (corresponding to the cytoplasmic tail of Melan-A) immobilized on glutathione beads with proteasome-depleted cytosol of melanoma cells, ATP, and biotinylated Ub (Figure 1E). Mono-ubiquitylation of Melan-A was confirmed by SDS elution of the GST-Melan-A_{50–118} fusion from the beads after the reaction, followed by immunoprecipitation and blotting with anti-Melan-A antibody (Figure 1E). We conclude that the integral membrane protein Melan-A undergoes ubiquitylation both in melanoma and nonmelanocytic cells.

Nonubiquitylated Melan-A Is More Stable and Accumulates on Melanosomal/Lysosomal Organelles

Because Ub plays a central role in protein degradation, we next investigated the role of ubiquitylation on Melan-A stability. Melan-A-negative SK-Mel-37 melanoma cells were transduced with recombinant lentiviruses encoding wild-type Melan-A or Melan-A^{K1-6R}. The metabolic stability of these proteins was analyzed by pulse chase. Whereas the half-life of wild-type Melan-A was ~ 4 h, Melan-A^{K1-6R} was barely degraded over the first 3 h of chase and $>60\%$ remained after 5 h (Figure 2A). A similar stabilization also was observed with an HA-tagged version of Melan-A^{K1-6R} transduced into Melan-A-positive MNT-1 cells compared with a similarly transduced HA-tagged wild-type Melan-A, as well as to the endogenous Melan-A expressed in these cells (Figure 2B). Interestingly, in both SK-Mel-37 and MNT-1 cells, Melan-A^{K1-6R} still underwent some degradation in spite of the fact that the N terminus of the protein (the only remaining potential Ub acceptor) (Ikeda *et al.*, 2002) is not exposed to the cytosol. This observation suggests the existence of a Ub-independent proteolysis of Melan-A. Melan-A degradation was further analyzed by following the steady-state levels of the wild-type and mutated proteins upon treatment of transduced SK-Mel-37 cells with the protein synthesis inhibitor cycloheximide. The results confirmed a greater stability of Melan-A^{K1-6R} compared with its wild-type form (Figure 2C).

Figure 1. Melan-A is a ubiquitylated protein. (A) Analysis of Melan-A immunoprecipitates from MNT-1 melanoma cells. Lysates from MNT-1 melanoma cells transduced (+) or not (-) with HA-tagged Melan-A were precipitated with anti-HA antibody. Immunocomplexes were resolved by SDS-PAGE and detected by Colloidal Blue staining. Arrows indicate the three protein bands that were cut from the gel and analyzed by mass spectrometry. (B) Detection of Ub in Melan-A immunoprecipitates from melanoma cells. MNT-1 cells were lysed by boiling in SDS (SDS +) followed by dilution with a Triton X-100 buffer, or directly in the latter buffer (SDS -). Lysates were immunoprecipitated with control (c, anti-CD8) or anti Melan-A (A103) antibody. Immunocomplexes were resolved by SDS-PAGE and detected by Western blotting with anti-Ub antibody. An aliquot of immunoprecipitates was used to detect Melan-A with antibody M2-7C10. (C) In vivo ubiquitylation assay in HEK293T cells. Cells were cotransfected with plasmids encoding Melan-A and HA-tagged Ub as indicated. Lysates of transfected cells were immunoprecipitated with anti-Melan-A antibody and analyzed by Western blotting. Parallel blots were probed with anti-HA and anti-Melan-A antibodies, as indicated. Total incorporation of Ub in cellular proteins was detected in total lysates (right). (D) In vivo ubiquitylation assay of Melan-A lysine mutants. HEK293T cells were cotransfected with plasmids encoding HA-tagged Ub and the indicated Melan-A proteins. Lysates of transfected cells were immunoprecipitated with anti-Melan-A antibody and analyzed as in C. (E) In vitro ubiquitylation assay. GST-Melan-A immobilized on glutathione beads was incubated for the indicated times with proteasome-depleted cytosol and biotinylated Ub, in the presence or absence of ATP. The material released from the beads was subjected to Western blot analysis with horseradish peroxidase (HRP)-conjugated streptavidin (SA). Right, GST-Melan-A was released from the beads, immunoprecipitated with anti-Melan-A antibody A103, and subjected to Western blot analysis with HRP-conjugated SA. The arrow points to ubiquitylated GST-Melan-A.

Immunofluorescence confocal analysis was performed to determine the specific subcellular compartment where Melan-A^{K1-6R} accumulates. When expressed in Melan-A-negative SK-Mel-37 cells, wild-type Melan-A was mainly detected in the Golgi area, with some cells additionally displaying a punctated pattern (Figure 3A, top), similar to what we observed after transfection of other Melan-A negative, a-pigmented cells (Rimoldi *et al.*, 2001). In contrast, a strong punctated pattern was predominant in SK-Mel-37 cells expressing Melan-A^{K1-6R} (Figure 3A, bottom). Some background fluorescence was also visible over the entire cytoplasm. Double immunofluorescence analyses showed the Melan-A-positive vesicular structures (both in wild-type Melan-A and Melan-A^{K1-6R}-transduced cells) were also positive for Lamp-1, a marker of late endosomal/lysosomal compartments (Figure 3A, insets). Because SK-Mel-37 cells do not express tyrosinase and gp100/Pmel17 and are thus likely to lack melanosomes, we investigated the localization of Melan-A^{K1-6R} in

pigmented MNT-1 cells. Immunofluorescence with anti-HA antibodies was performed to detect HA-tagged wild-type Melan-A or Melan-A^{K1-6R} in transduced cells. As reported previously, endogenous Melan-A was localized in the Golgi region and vesicular structures throughout the cytoplasm, characterized as melanosomes of different maturation states, particularly immature (Figure 3B, left; De Maziere *et al.*, 2002). A similar localization was observed with the exogenous HA-tagged wild-type protein (Figure 3B, middle), although the latter displayed a more important vesicular component extending to the cell periphery. Melan-A^{K1-6R} showed a predominant accumulation in organelles distributed along the rim of cells (Figure 3B, right). Double immunofluorescence with lysosomal and melanosomal markers showed that the Melan-A^{K1-6R} staining pattern mostly resembled that obtained for TRP-1, a protein of mature melanosomes (Figure 3C) (Raposo *et al.*, 2001). Indeed, a high degree of colocalization was obtained with this marker. Some colocalization

Figure 2. Increased stability of Melan-A^{K1-6R}. (A) Melan-A negative SK-Mel-37 cells were transduced with wild-type Melan-A and Melan-A^{K1-6R}. Cells were pulsed with ³⁵S-Cys and chased for the indicated times. Melan-A was precipitated from lysates with antibody A103. Autoradiography shows a representative experiment. Quantitative analysis (bottom) shows the mean and SD from three experiments and SD. (B) MNT-1 cells transduced with HA-tagged wild-type Melan-A [wt (exog.)] or Melan-A^{K1-6R} (K1-6R) were subjected to pulse chase as in A. Anti-HA antibody was used to precipitate the exogenously expressed Melan-A proteins [wt (exog.) and K1-6R]. Endogenous Melan-A [wt (endog.)] was subsequently precipitated with A103 antibody from the HA-immunodepleted lysates. Autoradiography shows a representative experiment. Quantitative analysis (bottom) shows the mean and SD from three experiments. (C) Analysis of Melan-A stability in cells treated with cycloheximide. SK-Mel-37 cells transduced with wild-type Melan-A or Melan-A^{K1-6R} were treated with 50 μ M cycloheximide (CHX). Lysates from cells collected at the indicated time points were subjected to SDS-PAGE and Western blot analysis with anti-Melan-A or anti-actin antibodies.

also was observed with gp100, which is detected primarily in stage I and II melanosomes (Raposo *et al.*, 2001), and Lamp-1, but the frequency of double-stained structures was lower than with TRP-1 (Figure 3C). We conclude from these experiments that the ubiquitylation of Melan-A may be required for the sorting of Melan-A into lysosomes for degradation.

Melan-A Is Degraded by Both the Lysosome and Proteasome

In a previous work, we demonstrated that at least a fraction of Melan-A is degraded by the proteasome, because the production of the antigenic peptide recognized by Melan-A-specific cytolytic T lymphocytes was abolished upon proteasome inhibition (Rimoldi *et al.*, 2001). However, the accumulation of Melan-A^{K1-6R} in lysosomal-related organelles described above prompted us to test whether Melan-A also is degraded by lysosomes. MNT-1 melanoma cells were metabolically labeled with ³⁵S-Cys, and the extent of Melan-A degradation was monitored after a 7-h chase period in the absence or presence of various protease inhibitors (Figure 4A). In the presence of the lysosomotropic agents chloroquine and NH₄Cl, the degradation of Melan-A was significantly reduced. The proteasome inhibitor lactacystin also increased the stability of Melan-A, confirming our previous results. The cysteine protease and proteasome inhibi-

tor MG132 stabilized most efficiently Melan-A. Melan-A levels in cells treated with the various inhibitors also were analyzed by Western blotting. In MNT-1 cells, inhibitors of lysosomal functions (chloroquine, NH₄Cl, and the vATPase inhibitors concanamycin A and bafilomycin A) as well as proteasomal inhibitors induced Melan-A accumulation (Figure 4B). In contrast, little effect was observed with the same drugs on Melan-A^{K1-6R} exogenously expressed in MNT-1 cells (Figure 4B), indicating that the stabilization is linked to ubiquitylation. Finally, in Melan-A-transduced SK-Mel-37 cells treated with NH₄Cl or chloroquine, a marked accumulation of Melan-A was observed on Lamp-1-positive compartments (see online supplemental material). Based on these results, it seems that a significant fraction of Melan-A is degraded by lysosomal enzymes in melanoma cells.

Melan-A Interacts with the E3 Ub Ligases NEDD4 and Itch

Having demonstrated the contribution of the ubiquitylation of Melan-A to its turnover, we sought to identify the Ub ligase (E3) responsible for this process. Several E3 have been described that specifically ubiquitylate cell surface proteins destined for lysosomal degradation. In mammals, NEDD4, Itch, and Cbl seem to play a predominant role in this process (Ingham *et al.*, 2004; Marmor and Yarden, 2004). To determine whether Melan-A interacts with one of these E3, en-

Figure 3. Confocal immunofluorescence analysis of Melan-A^{K1-6R}-transduced melanoma cells. (A) SK-Mel-37 cells were transduced with wild-type Melan-A (Melan-A w.t.) or Melan-A^{K1-6R} and double labeled with anti-Melan-A antibody A103 (red fluorescence, left) and anti-Lamp antibody (green fluorescence, middle). Yellow in the merged images (right) indicates overlapping of staining. Insets show boxed

ogenous Melan-A was immunoprecipitated from MNT-1 melanoma cells and tested for association with E3 by Western blotting by using antibodies specific for NEDD4 or Itch. As can be observed in Figure 5A, both NEDD4 and Itch coimmunoprecipitated with Melan-A. We were unable to detect Melan-A in Itch and NEDD4 immunoprecipitates (Figure 5A). This could be partly due to the fact that the E3 ligases could not be immunoprecipitated quantitatively by their respective antibodies in these experiments. Small amounts of Itch and NEDD4 also seemed to coimmunoprecipitate reciprocally. The association of Melan-A with the two HECT-E3 ligases was further confirmed in cotransfection experiments in HEK293T cells. In this system, NEDD4 and Itch were readily detected in the immunocomplexes precipitated by an anti-Melan-A antibody only in the presence of transfected Melan-A (Figure 5B). No interaction between Melan-A and c-Cbl, a RING domain Ub ligase involved in membrane protein degradation (Marmor and Yarden, 2004), could be detected either by coimmunoprecipitation in melanoma cells (Figure 5A) or by a GST pull-down assay with GST-Melan-A (Figure 5C).

We then investigated whether NEDD4 or Itch, or both, were involved in ubiquitylating Melan-A by using the HEK293T *in vivo* ubiquitylation system. Overexpression of NEDD4 increased the ubiquitylation of Melan-A in a dose-dependent manner, concomitant with a small decrease in the unmodified protein (Figure 6A). In contrast, overexpression of an enzymatically inactive form of NEDD4 (NEDD4^{C867S}) did not affect Melan-A ubiquitylation nor its total levels (Figure 6A). Surprisingly, overexpression of Itch led not only to a decreased level of ubiquitylated Melan-A but also to a marked decrease in nonubiquitylated Melan-A (Figure 6B). Overexpression of the enzymatically inactive Itch^{C830A} did not produce this effect (Figure 6B). To assess whether NEDD4 indeed ubiquitylates Melan-A, we performed an *in vitro* ubiquitylation assay in the presence of cell extracts immunodepleted or not of NEDD4. As shown in Figure 6C, the band corresponding to ubiquitylated Melan-A was greatly reduced upon depletion of NEDD4, suggesting that Itch, which was present in the cytosol extract, did not ubiquitylate Melan-A.

To further substantiate the physiological significance of the interaction of Melan-A with NEDD4 and Itch, we silenced the endogenous expression of the two enzymes in melanoma cells. Melan-A transduced SK-Mel-37 cells transfected with siRNA duplexes specific for NEDD4 or Itch, but not a control siRNA sequence, showed increased Melan-A levels, paralleled by a slight reduction in ubiquitylated Melan-A forms (Figure 7A). Conversely, overexpression of NEDD4 or Itch in the same cells was accompanied by a marked reduction of Melan-A in the transfected cells, as shown by immunofluorescence analysis (Figure 7B). Because the overexpression of NEDD4 and Itch in HEK293T cells affect the ubiquitylation and degradation of Melan-A

Figure 4. Effect of proteasome and lysosome inhibitors on Melan-A stability. (A) MNT-1 melanoma cells were pulsed with ³⁵S-Cys and chased for 7 h in the absence or presence of the indicated drugs. Appropriate zero time chase points for different treatments were set up as detailed in *Materials and Methods*. Melan-A was precipitated from lysates and analyzed by SDS-PAGE and autoradiography/PhosphorImager analysis. Quantitative analysis was performed with results from three experiments. (B) MNT-1 and Melan-A^{K1-6R} transduced MNT-1 cells were incubated for 5 h with MG132 (50 μ M) and lactacystin (50 μ M) or 7 h with NH₄Cl (30 mM), chloroquine (100 μ M), concanamycin A (Con. A; 20 nM), and bafilomycin A (Bafil. A; 100 nM). Cell lysates were prepared and analyzed by Western blotting with anti-Melan-A and anti-HA antibodies to detect the endogenous (MNT-1) or the transduced (MNT-1/Melan-A^{K1-6R}) Melan-A, respectively.

differently and the reduced expression of either of the two E3 ligases stabilizes Melan-A in melanoma cells, it seems that NEDD4 and Itch participate in a cooperative manner to the degradation of Melan-A.

DISCUSSION

In this work, we have provided evidence that the melanosomal protein Melan-A is ubiquitylated in melanoma cells. This modification provides a signal for the lysosomal degradation of Melan-A. We have identified two members of the HECT-E3 ligase family that are involved in this process. In pigmented cells, Melan-A lacking ubiquitylation sites accumulated in melanosomes. These results suggest that the function of Melan-A is regulated primarily by the Ub-lysosomal degradation machinery.

Figure 3 (cont). areas at higher magnification. (B) Localization of Melan-A in MNT-1 cells transduced with control lentivirus [indicated as C (-)], HA-tagged wild-type Melan-A (Melan-A w.t.), or HA-tagged Melan-A^{K1-6R} (indicated as Melan-A^{K1-6R}). Endogenous and transduced Melan-A were detected with antibody A103 or anti-HA antibody, respectively. (C) Double immunofluorescence was performed on MNT-1 cells transduced with HA-tagged Melan-A^{K1-6R} by using anti-HA antibody and anti-TRP-1 (mature melanosomes), anti-gp100 (early melanosomes), or anti-Lamp-1 (late endosomes/lysosomes) antibodies. High-magnification images show melanoma dendrites. Single plane confocal images are shown. Bars, 10 μ m.

Figure 5. Interaction of Melan-A with NEDD4 and Itch. (A) Lysates of MNT-1 cells were immunoprecipitated (IP) with anti-Melan-A (A103), NEDD4, Itch, c-Cbl, or control (c; anti-CD8) antibodies. Immunocomplexes were analyzed by Western blotting as indicated. An aliquot of total cellular lysate was loaded on the gels for comparison (equivalent to ~1% of the immunoprecipitated material for NEDD4, c-Cbl, and Melan-A and 10% for Itch). (B) HEK293T cells were transfected with Melan-A, NEDD4, and Itch encoding plasmids in the indicated combinations. Cell lysates were precipitated with control (anti-CD8; c) or anti-Melan-A antibodies. Immunocomplexes were analyzed by Western blotting with the indicated antibodies. (C) GST-Melan-A was incubated with a lysate of HEK293T cells and then recovered with glutathione beads. The material associated with the beads was released and analyzed by Western blotting with the indicated antibodies. Control incubations were performed with GST alone. An aliquot of total cellular lysate was also loaded on the gel for comparison. PD, pull-down.

A major effect of ubiquitylation on Melan-A was on its rate of degradation, as the mutant lacking lysine residues (Melan-A^{K1-6R}), to which Ub cannot be transferred, was more stable than the wild-type protein. Both the proteasome and lysosomal enzymes seem to participate in Melan-A degradation, as shown using acidotropic drugs and proteasome inhibitors. The predominance of the lysosomal pathway is highlighted by the striking accumulation of Melan-A^{K1-6R} in Lamp-1-positive vesicles in melanoma cells lacking a pigmentation program. In fully differentiated pigmented melanoma cells, the same Melan-A mutant rather accumulated on mature melanosomes. In previous electron microscopy studies of pigmented cells, we observed that Melan-A was progressively lost from the limiting membrane of maturing melanosomes, with a concomitant partial shift to internal compartments. In addition, Melan-A was observed in internal vesicles of multivesicular bodies (MVBs) and lysosomes (De Maziere *et al.*, 2002). Together with the current findings, these observations suggest a ubiquitylation-dependent sorting of Melan-A from the surface of early stage melanosomes to lysosomes. Together, our results strengthen the hypothesis that proteins are actively sorted from maturing melanosomes to lysosomes (Raposo *et al.*, 2001; Raposo and Marks, 2002).

Ubiquitylation, and more particularly monoubiquitylation, is emerging as a major signal for sorting transmembrane proteins to intraluminal vesicles of MVBs for subsequent degradation in the lysosome (Katzmann *et al.*, 2002; Raiborg *et al.*, 2003). Although several examples of cells surface receptors targeted to MVBs for lysosomal degradation

via a Ub-dependent mechanism are known, Melan-A is, to our knowledge, the first example of a mammalian membrane protein located in a cytoplasmic compartment to follow this fate. In yeast, the vacuolar hydrolase carboxypeptidase S (CPS) is sorted to the luminal vesicles of MVBs via a Ub-dependent mechanism (Katzmann *et al.*, 2001). It is therefore tempting to speculate that monoubiquitylation is a more general MVB sorting mechanism for cellular membrane proteins. It should be noted that the transport of Melan-A from the TGN to endosomes/melanosomes of mammalian cells, as shown in Figure 3, seems to occur independently of ubiquitylation.

Melan-A associated in melanoma cells with two E3 Ub ligases of the NEDD4 family, namely, NEDD4 and Itch. This interaction seems to be functionally relevant, because silencing of the individual E3 Ub ligases affected Melan-A levels. NEDD4 and Itch contain two to four WW protein-protein interaction domains, characterized by the presence of two highly conserved TRP residues. The principal target sequence of WW domains is the so-called PY motif (PPXY). Other motifs, such as PPLP and phosphorylated serine/threonine residues also have been shown to mediate the binding to WW domains (Macias *et al.*, 2002). Examination of Melan-A sequence reveals that the C-terminal cytoplasmic region contains two putative PY motifs (PPAY and PPPY). Preliminary experiments indicate that this region is indeed implicated in Melan-A binding to the E3 ligases. It is noteworthy that the Melan-A PY motifs are highly conserved from zebrafish to human (our unpublished data) and therefore represent a crucial feature of the protein. Smad1, Crk-L,

Figure 6. Effect of NEDD4 and Itch on Melan-A ubiquitylation. (A) HEK293T cells were cotransfected with plasmids encoding Melan-A, HA-tagged Ub, NEDD4, or a catalytically inactive form of NEDD4.1 (NEDD4-1/CI), as indicated. Two amounts of NEDD4 plasmid were used (+, 0.2 μ g; ++, 2 μ g). Melan-A was immunoprecipitated from cell lysates, and immunocomplexes were analyzed by SDS-PAGE and Western blotting. Parallel blots were used to detect Ub (anti-HA antibody), and Melan-A. NEDD4 expression was analyzed by Western blotting of total cell lysates. (B) HEK293T cells were cotransfected with plasmids encoding Melan-A, HA-tagged Ub, Itch, or a catalytically inactive form of Itch (Itch/CI), as indicated. Two amounts of Itch plasmid were used (+, 0.2 μ g; ++, 2 μ g). Ubiquitylation of Melan-A was analyzed by Western blotting of immunoprecipitates as in A. (C) Ubiquitylation of Melan-A depends on NEDD4. Immobilized GST-Melan-A was incubated with proteasome-depleted cytosol in the presence of biotinylated Ub for the indicated times. After the reaction, GST-Melan-A was released from the beads, immunoprecipitated with anti-Melan-A antibody, and subjected to Western blot analysis with horseradish peroxidase-conjugated streptavidin (SA). Where indicated, NEDD4 was immunodepleted from the cytosol before the reaction. Control depletion was performed with an irrelevant antibody.

Figure 7. Knockdown and overexpression of NEDD4 and Itch affect Melan-A levels in melanoma cells. (A) Melan-A transduced SK-Mel-37 cells were transiently transfected with siRNA duplexes specific for NEDD4, Itch, or a control siRNA sequence. Total cell lysates were analyzed by Western blotting with the indicated antibodies. An aliquot of lysates was immunoprecipitated with anti-Melan-A antibody before detection with anti-Ub antibody. Approximate size of the ubiquitylated Melan-A species is indicated. (B) Melan-A transduced SK-Mel-37 cells were transfected with Itch and NEDD4 encoding plasmids. Neomycin enriched transfectants were analyzed by double staining with the indicated antibodies and immunofluorescence microscopy. Endogenously expressed Itch and NEDD4 were below detection limit under the conditions the pictures were acquired. Bar, 50 μ m.

and the EBV-encoded LMP2A are among the few proteins that have been reported to interact *in vivo* with at least two members of the same family of ligases (Elly *et al.*, 1999; Ikeda *et al.*, 2000; Lin *et al.*, 2000). In T cells, ubiquitylation of Bcl10 was recently reported to be promoted either by NEDD4 or Itch (Scharschmidt *et al.*, 2004). In both HEK293T cells and melanoma cells, the association of Melan-A with NEDD4 and Itch resulted in decreased Melan-A levels. Interestingly, in HEK293T cells the overexpression of NEDD4 led to a concomitant increase in ubiquitylated Melan-A. Instead, overexpression of Itch accelerated the degradation of Melan-A. It is therefore possible that these two E3 ligases perform complementary functions. One interesting possibility is that NEDD4 initiates the ubiquitylation of Melan-A followed by the interaction with Itch, which in turn promotes the segregation of ubiquitylated Melan-A from the limiting membrane to internal vesicles and subsequent degradation. However, other interpretations are compatible with our results, and the exact role of the two E3 ligases in the sorting and degradation pathway of Melan-A still has to be elucidated. The distinct activities of the two ligases also could be associated with their presence in different membrane microdomains of early endosomes/melanosomes and/or their interaction with distinct subsets of proteins. In this regard, it is noteworthy that Itch but not NEDD4 has been found to associate with a protein involved in membrane trafficking (Lindholm and Lévy, unpublished data). Moreover, it has been reported that the endocytosed chemokine receptor CXCR4 colocalizes in endosomes with the Ub-binding protein Hrs and Itch, but not NEDD4 (Marchese *et al.*, 2003).

Hrs and other Ub interacting motif-containing proteins play a central role in the sorting to MVBs by bridging ubiquitylated proteins to the multiprotein complex ESCRT machinery, which promotes inward vesiculation (Raiborg *et al.*, 2003; Gruenberg and Stenmark, 2004). Hrs associates with clathrin in typical bilayered coat structures found primarily in early endosomes (coated endosomes) (Raiborg *et al.*, 2002; Sachse *et al.*, 2002). This places these structures at the center of the endosomal sorting machinery. Coated endosomes have been identified in different cell types. In melanocytic cells, they have been proposed as the most immature melanosomal structure (stage I), because they carry Melan-A and the early melanosomal marker gp100, a limiting membrane protein that, after enzymatic release of its luminal domain, initiates the fibril formation within the organelle (Berson *et al.*, 2001; Raposo *et al.*, 2001; Berson *et al.*, 2003). Interestingly Melan-A has been shown to be enriched in the bilayered coat (De Maziere *et al.*, 2002). Thus, the coated endosome/early melanosome is a candidate intermediate where the active sorting of Melan-A to internal vesicles can occur (the coat was not observed in stage II–IV melanosomes (Raposo *et al.*, 2001)).

In conclusion, our study shows that ubiquitylation of a melanosomal protein affects its sorting and degradation and could therefore impact on the physiology of pigmented cells.

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